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Short communication

Effects of spiramine T on antioxidant enzymatic activities and nitric oxide production in cerebral ischemia–reperfusion gerbils

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Abstract

Spiramine T, an atisine-type diterpene alkaloid isolated from the Chinese herbal medicine *Spiraea japonica* var. *acuta* (Rosaceae), was shown to have neuroprotective effects on cerebral ischemia–reperfusion injury. In this study, the effects of spiramine T on antioxidant enzymes and nitric oxide production were evaluated in gerbils subjected to global forebrain ischemia (10 min) and reperfusion (5 days). Spiramine T (1.0 and 2.0 mg kg^{-1} i.p.) markedly reduced the content of lipid peroxide (LPO), increased the glutathione peroxidase (GSH-PX) activity, and inhibited the increase of nitric oxidase (NOS) activity and nitric oxide production in the cortex during ischemia–reperfusion in gerbils. These results suggested that the neuroprotective effects of spiramine T were related to modulation of endogenous antioxidant enzymatic activities and reduction of the formation of nitric oxide.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Spiraea japonica var. *acuta*; Rosaceae; Spiramine T; Cerebral ischemia–reperfusion; Antioxidant enzyme; Nitric oxide synthase

Cerebral ischemia, particularly the period following reperfusion is accompanied by the enhanced-formation of reactive oxygen species in brain tissue. Excessive production of reactive oxygen species such as superoxide anion, hydroxyl radical, and hydrogen peroxide, and nitric oxide (NO) has been shown to play a critical role in the development of ischemia–reperfusion injury [5,19]. Those reactive oxygen species may induce cerebral damage either directly through interacting and destroying cellular proteins, lipids and DNA, or indirectly by affecting normal cellular signaling pathways and gene regulation [21]. Reactive oxygen species can be scavenged by endogenous antioxidants, including superoxide dismutase (SOD) that catalyses the dismutation of the superoxide anion, glutathione peroxidase (GSH-PX) and catalase (CAT) that mediate the breakdown of hydrogen peroxide [6,7]. In addition, many recent studies have examined the role played by nitric oxide in cerebral ischemia. It has been shown that activation of the *N*-methyl-D-aspartate (NMDA) receptor also activates NO synthase (NOS), which leads to excess production of $NO^{\cdot}$ [13]. High concentrations of $NO^{\cdot}$ are toxic and interact with superoxide anion to produce the highly toxic peroxynitrite anion [21]. Therefore, it is beneficial for the treatment of ischemic diseases to maintain the activities of antioxidant enzymes and to inhibit NOS activity.

Spiraea japonica L. (Rosaceae) and its varieties are widespread in Yunnan Province, People's Republic of China. The young leaves, fruits, and roots of some of those plants have been used as diuretic, detoxicant, and analgesic agents and for the treatment of inflammation, cough, headache, and toothache in traditional Chinese medicine [24,28]. Previous chemical investigations on *Spiraea japonica* and its varieties have led to the report of seven new atisane-type diterpenoids [15] and 30 new diterpene alkaloids of atisine- and hetisine-type [8,16,22]. Spiramine

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Q, an antisine-type diterpene alkaloid from *S. japonica* var. *incisa* was shown to have selective inhibition against rabbit platelet aggregation induced by arachidonic acid (AA) in vitro and ex vivo and decreased the mouse mortality caused by intravenous injection of AA, and more active than aspirin [18]. Moreover, the neuroprotective effects of the 95% ethanol (EtOH) extract of *Spiraea japonica* var. *acuta* on cerebral ischemia–reperfusion injury produced by 10 min occlusion of the bilateral common carotid arteries, followed by 5 days reperfusion in gerbils, were shown by enhancing the recovery of encephalogram (EEG) amplitude during reperfusion, and more active than *Ginkgro Biloba* Extract (EGb 761). Bioactivity-guided fractionations revealed that the basic and neutral fractions from this extract were active. The neutral fraction was neuroprotective, possibly due to the antioxidative action of its flavonoid components as those in EGb761. Further studies indicated that intravenous spiramine T (Fig. 1), an atisine-type diterpene alkaloid from the basic fraction, markedly reduced the stroke index, and enhanced the recovery of EEG amplitude during reperfusion and decreased the concentrations of cortex calcium and lipid peroxide (LPO) in a dose-dependent manner [12]. In the present study, we focus on its effects on antioxidant enzymatic activities and nitric oxide production after cerebral ischemia–reperfusion in gerbils.

The roots of *S. japonica* var. *acuta* (Rosaceae) were collected in Lijiang, Yunnan Province, PR China, and identified by Professor H. Sun at Kunming Institute of Botany, Chinese Academy of Sciences, and the voucher specimen is deposited in the Herbarium of Kunming Institute of Botany under No. 97012. Spiramine T (purity >95% by HPLC) was isolated from the basic portion of the 95% ethanol extract as previously described [8,22]. It was dissolved in 0.1 mol l^{-1} aqueous HCl. The solution was adjusted to pH 7.0 with 0.1 mol l^{-1} aqueous Na_2CO_3 before administration.

The adult gerbils, half male and half female, weighting 50–70 g (obtained from Yunnan Pharmacological Laboratories of Natural Products), were anesthetized with sodium pentobarbital (50 mg kg^{-1} i.p.) and the bilateral common carotid arteries were occluded with atraumatic arterial clips for 10 min followed by 5 days reperfusion. In sham-operated gerbils, the procedure was the same as the above, except for the occlusion of the bilateral common carotid arteries. Body temperature of animals was maintained at 36.5–37.5 °C with a heating lamp during anesthesia. Spiramine T (1.0 and 2.0 mg kg^{-1}), nimodipine 1.0 mg kg^{-1} or saline (for the sham-operated group (Sham) and ischemia–reperfusion group (Isc–Rep)) were administered intraperitoneally (i.p.) 20 min prior to ischemia and once daily for 5 days after reperfusion. At the end of experiment, gerbils were decapitated, and their forebrain cortex was removed quickly and stored at −30 °C. On the day of assay, each cerebral cortex was defrosted and homogenized with a buffer consisting of 10 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris–HCl and 0.1 mmol l^{-1} EDTA (pH 7.4). The homogenate was centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant was used for bioassays. The concentration of the protein in the supernatant was measured by the method of Bradford [1].

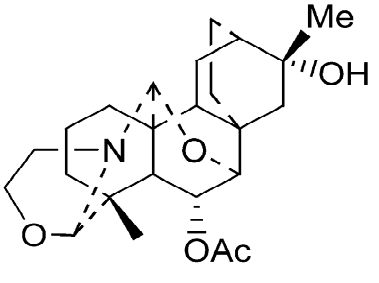

Fig. 1. Structure of spiramine T.

The content of LPO in the supernatant was measured with thiobarbituric acid as described previously [17]. The level of LPO was expressed as nmol mg^{-1} protein of malondialdehyde (MDD) using 1,1,3,3-tetramethoxypropane as external standard. The activities of antioxidant enzymes were measured using commercially available kits (Jiancheng Bioengineering). The assay for SOD activity was based on its ability to inhibit the oxidation of oxymine by $O_2^{-\cdot}$ produced from the xanthine–xanthineoxiase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The assay for GSH-PX activity was determined by quantifying the rate of oxidation of the reduced glutathione (GSH) to the oxidized glutathione (GSSG) by H_2O_2 catalyzed by GSH-PX. One unit of GSH-PX was defined as the amount that reduced the level of GSH by 1 μmol l^{-1} in 1 min per mg protein. The assay for CAT activity was based on its ability to decompose H_2O_2. One unit of CAT was defined as the amount that reduced the level of H_2O_2 by 1 μmol l^{-1} in 1 min per mg protein. Nitric oxide content was determined as previously described by Tracey et al. [20]. NOS activity was measured by the method previously reported [11], and expressed in unit as 1 nmol of NO formed in 1 min per mg protein.

Data are expressed as mean±S.D. Statistical analysis was carried out using one-way ANOVA followed by Newman–Keuls multiple comparison test using $P<0.05$ as the level of significance.

The significant increment of MDA content in the cortex was observed in the Isc–Rep group as compared with the sham-operated group ($P<0.01$). Being treated with spiramine T, the MDA content drastically decreased, which was similar to nimodipine (Fig. 2). In the Isc–Rep group, the activity of CAT was elevated ($P<0.05$, compared with the sham-operated group); the activities of SOD and GSH-PX slightly decreased, but failed to reach significance. Spiramine T significantly increased the GSH-PX activity in the cortex after ischemia–reperfusion injury, while no effects of spiramine T on the activities of SOD and CAT

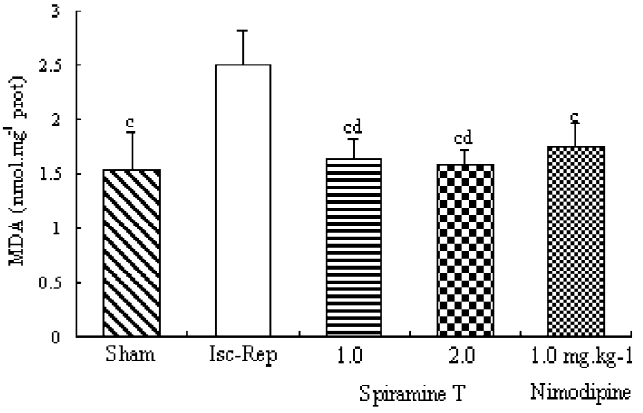

Fig. 2. Effects of intraperitoneal spiramine T on MDA content in the cortex after 5 days reperfusion in gerbils. Data represent mean±S.D., $n=6$ gerbils. $^{c}P<0.01$ versus Isc–Rep group; $^{d}P>0.05$ versus nimodipine group. One-way ANOVA followed by Newman–Keuls multiple comparison test.

were observed. Nimodipine markedly increased SOD activity, while it had no effects on GSH-PX and CAT activities (Fig. 3).

The NOS activity and NO production clearly increased under ischemia–reperfusion injury. But they were significantly reduced by treatment with spiramine T in both 1.0 and 2.0 mg kg^{-1} groups as compared to the Isc–Rep gerbils ($P<0.01$), and were lower than that of the sham-operated group. Nimodipine also decreased the NOS activity and NO production, but the inhibition was less potent than that of spiramine T (Fig. 4).

The present study demonstrated that intraperitoneal injection of spiramine T attenuated the increase of the MDA content of the cortex induced by ischemia–reperfusion, suggesting that spiramine T had the potential to protect neuronal membranes from lipid peroxidative damage. This finding was consistent with our previous report showing its neuroprotective effects on acute forebrain ischemia–reperfusion injury in gerbils [12].

Reactive oxygen species were believed to play a central role in ischemia–reperfusion injury to neurons. The brain is exceptionally vulnerable to the cytotoxic effects of reactive oxygen species [14]. In oxidative stress, superoxide anion and hydrogen peroxide formed during ischemia–reperfusion cannot be readily scavenged because of the low activities of CAT, SOD, and GSH-PX presented in the brain. Moreover, brain membrane lipids are very rich in polyunsaturated fatty acids, which are especially sensitive to free radical-induced lipid peroxidation [3]. SOD and GSH-PX are considered to be the major endogenous antioxidants in the brain and have been demonstrated to be the most important enzymes for neuronal defense against peroxide toxicity in vitro [4]. GSH-PX involved in the reduction of cytosolic hydrogen peroxide and SOD catalyzes the dismutation of superoxide anion. Overexpression of endogenous SOD and GSH-PX has been demonstrated to protect against ischemia–reperfusion damage in the

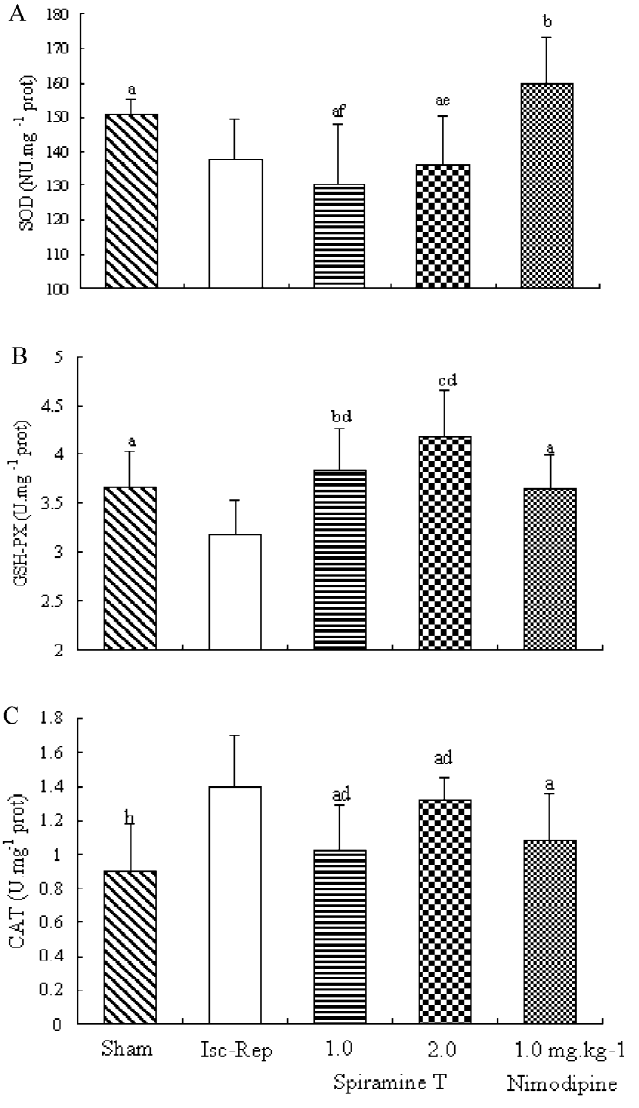

Fig. 3. Effects of intraperitoneal spiramine T on activities of antioxidant enzymes in the cortex after 5 days reperfusion in gerbils. Data represent mean±S.D., $n=6$ gerbils. $^{a}P>0.05$, $^{b}P<0.05$, $^{c}P<0.01$ versus Isc–Rep group; $^{d}P>0.05$, $^{e}P<0.05$, $^{f}P<0.01$ versus nimodipine group. One-way ANOVA followed by Newman–Keuls multiple comparison test.

brain [23,25]. Islekel [10] has demonstrated that the activities of SOD and GSH-PX, the major antioxidant enzymes, decreased in the brain after ischemia–reperfusion, while the activity of CAT increased, suggesting that a disturbance in endogenous oxidant–antioxidant balance occurred in ischemia–reperfusion. Our study showed that the activities of SOD and GSH-PX in the cortex had the tendency to decrease after ischemia–reperfusion, while the activity of CAT increased in the present ischemia–reperfusion condition. Spiramine T, when administered to gerbils, significantly elevated the GSH-PX activity in both dose groups, which was different from nimodipine which increased the SOD activity. Obviously, spiramine T scavenged hydrogen peroxide mainly by increasing the activity of GSH-PX; thus, further decreasing the formation

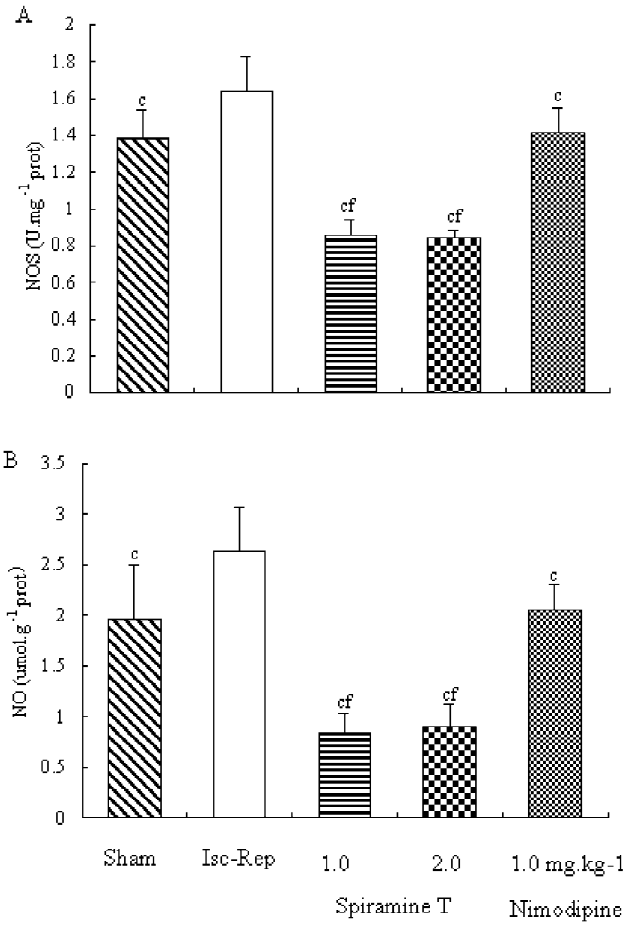

Fig. 4. Effects of intraperitoneal spiramine T on NOS activity (A) and NO production (B) in the cortex after 5 days reperfusion in gerbils. Data represent mean±S.D., $n=6$ gerbils. $^{c}P<0.01$ versus Isc–Rep group; $^{f}P<0.01$ versus nimodipine group. One-way ANOVA followed by Newman–Keuls multiple comparison test.

of hydroxyl radical and ROOH and attenuating lipid peroxidative damage after ischemia–reperfusion. However, it is not clear whether spiramine T induced the expression of endogenous antioxidant enzymes or protected them through inhibiting NOS activity.

During ischemia–reperfusion, excessive production of NO formed by neuronal NOS when stimulated by glutamate release or from inducible NOS may exacerbate ischemic injury by promoting oxidative damage and energy failure [9]. A number of recent studies have shown that NOS activity is increased in the rodent brain after global [2] and focal cerebral ischemia [26]. In this study, we also observed the increase of NOS activity after ischemia–reperfusion. Spiramine T inhibited the increase of the activity of NOS and the production of NO induced by ischemia–reperfusion. These results suggested that spiramine T attenuated the formation of NO by inhibiting NOS, which contributed to its neuroprotection. Moreover, it has been shown that the surgical procedure could lead to artificially high levels of inducible NOS in models of focal ischemia that involve surgical exposure of the middle cerebral artery [27]. The present study showed that the activity of NOS in spiramine T-treated groups was lower than that in the sham-operated group, suggesting that the increase of NOS activity induced by surgical procedure might occur in the model of global cerebral ischemia, and spiramine T could reduce the NOS activity to normal level.

A previous study showed that the ability of spiramine T to attenuate calcium overloading was nearly equal to nimodipine [12]. A recent study has shown that nimodipine protected the brain from ischemic damage by inhibiting NOS activity resulting from the decrease in Ca^{2+} level in neurons [29]. Our research suggested that spiramine T protected the brain from ischemic damage by a different mechanism, because the effect of spiramine T in inhibiting NOS activity was much stronger than that of nimodipine.

In summary, the activities of SOD, GSH-PX and CAT were differently affected by ischemia–reperfusion injury. Spiramine T modulated the activities of endogenous antioxidant enzymes, and reduced the formation of nitric oxide by inhibiting the NOS activity. The abilities of spiramine T to increase the activity of GSH-PX and inhibit NOS activity under ischemia–reperfusion were attributed to its neuroprotective effects.

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